WILLARD, LAURA EVINS, M.S. Development and Analysis of Primary Cultures from the Midgut of the Honey Bee, *Apis mellifera*. (2012)
Directed by Dr. Olav Rueppell. 68pp.

Apis mellifera is an important research species of tremendous agricultural and economic importance. However, relatively few *in vitro* techniques are described for investigating honey bee physiology at the cellular or molecular level. This project describes a new technique for developing primary cell lines from the midgut of adult honey bees and characterizes the impact of donor age on the longevity, differentiation, and proliferation of intestinal stem cells within these cultures. Additionally, this study characterizes the effects of 20-hydroxyecdysone on intestinal stem cell proliferation and differentiation *in vitro*. Suspension cultures of midgut cells obtained through non-enzymatic dissociation were cultured in WH2 media with 1 µg/mL penicillin/streptomycin and 0.25 µg/mL Amphotericin B survived for up to 15 days. Age of donor had no significant effect on the longevity of cultures or rate of cell differentiation. However, the number of viable intestinal stem cells was significantly different between cultures from pre-eclosion pharate adults and cultures from workers. A 5-bromo-2'-deoxyuridine (BrdU) assay found very low levels of proliferation in cultures from all ages with the exception of foragers. Addition of 20-hydroxyecdysone did not affect the either longevity of the culture or the rate of cell differentiation or proliferation. These results suggest that the behavior of stem cells in culture is impacted by the age of the donor while differentiation is independent of age. Further, this work supports the idea that intestinal stem cells lose their proliferative capacity with age. The lack of effect of 20-hydroxyecdysone on measured parameters may indicate a physiological difference in the midguts of *Apis mellifera* and lepidopteran species and warrants further investigation. These cultures provide a starting point for future use as a model for honey bee midgut molecular and cellular physiology as well as *in vitro* studies of honey bee intestinal health.

DEVELOPMENT AND ANALYSIS OF PRIMARY CULTURES FROM THE MIDGUT OF THE HONEY BEE, *APIS MELLIFERA*

by

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A Thesis Submitted to
the Faculty of The Graduate School
at The University of North Carolina at Greensboro
in Partial Fulfillment
of the Requirements for the Degree
Master of Science

Greensboro
2012

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APPROVAL PAGE

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CHAPTER I

INTRODUCTION

Importance and biology of honey bees

The European honey bee, *Apis mellifera,* is an important research species. The honey bee has served as a model organism for studying social structure, aging, behavior, and other aspects of biology with multiple applications to animal and human health. This importance was underscored when the honey bee was the fifth insect species to have its genome sequenced in 2006, which opened up a multitude of new research possibilities. However, the majority of honey bee research has used *in vivo* or genetic techniques. There have been relatively few *in vitro* techniques developed for investigating the honey bee's physiology at the molecular or cellular level.

Apis mellifera is also of tremendous agricultural and economic importance. The honey bee is depended upon for the pollination of over 90 agricultural crops grown in the United States, with an estimated added economic value of over $15 billion annually (Colony Collapse Disorder Action Plan 2007). There are approximately 2.4 million managed honey bee colonies in the U.S., and the demand for apiculture continues to increase (Colony Collapse Disorder Action Plan 2007). In the winter of 2006/2007, beekeepers in 22 states reported colony losses of unknown origin in 30-90% of their hives (NRC Status of Pollinators Committee 2007). These losses were characterized by a disappearance of adult workers from the colony with an absence of dead bees found in or

near the hive (vanEngelsdorp 2009). Without workers present, the orphaned brood cannot survive and the colony collapses, hence this phenomenon has been termed Colony Collapse Disorder (CCD). Large-scale losses continued over the winters of 2007/2008 and 2008/2009 in the USA and Europe (vanEngelsdorp et al. 2009, 2010). These losses are potentially devastating to apiculture and agriculture, and threaten the world's food supply. Consequently, there is an urgent need for research into honey bee health.

CCD has been linked to multiple pathogens, such as Israeli acute paralytic virus, pesticides, and other environmental stresses, but ultimately the causes are still unknown (Cox-Foster 2007, Johnson et al. 2009). Recent research has shown a correlation between infections with the intestinal parasite *Nosema ceranae* and CCD symptoms (Higes et al. 2009) and changes in the abundance of rRNA transcripts in the digestive tracts of bees from collapsing colonies (Johnson et al. 2009). Both of these studies focused on the intestine because it is the primary interface between the honey bee and its environment and the target organ of many parasites and cytotoxins (Johnson et al. 2009). However, very little work has been done on the biology of the honey bee midgut, especially in adult bees. Further investigation into these processes would facilitate understanding of the biology underlying CCD and could potentially lead to effective treatments.

The honey bee has a social structure that involves three classes of bee, which all have different roles in the hive. The male honey bee is the drone. Drones develop from unfertilized eggs and are thus haploid, carrying only half the genetic information of the females. The sole function of the drone is to mate with queens from unrelated hives (Winston 1987). The sole queen is the only reproductive female in the hive. At the

beginning of her life, she mates with up to 45 drones and stores their sperm. For the remainder of her life, she focuses on reproduction and lays up to 2000 eggs per day (Rueppell et al. 2004). The non-reproductive females are referred to as worker bees and have a variety of roles within the hive. When workers emerge from their pre-eclosion pharate adults stage, they spend their first 24 hours cleaning the hive while their exoskeleton hardens, giving them the ability to fly and sting. They then become nurses and maintain the hive through feeding and caring for the young, and producing the comb. As workers age, they begin foraging for food, and storing that food in the form of pollen or honey (Winston 1987). Because workers make up the majority of the population of the hive and are solely responsible for supplying food, they are a natural starting point for any honey bee study.

Biology of the midgut

The honey bee (*Apis mellifera*) gut, which extends from the mouth to the anus, is one of the primary interfaces of the honey bee with its external environment. The main site of digestive activity in the gut is the midgut, which extends from the proventricular valve on the anterior end, separating it from the crop, to the rectum on the posterior end. The midgut is immediately surrounded by the excretory Malphigian tubules and the fat body. The tubular organ is composed of a highly folded single layer of cells attached to a thin basement membrane, which is composed of collagen, laminin, and fibronectin and provides an anchor for tracheae, nerves, and muscles (Lehane and Billingsley 1996). Within the gut, the semipermeable peritrophic matrix surrounds the lumen and protects

the underlying epithelial cells from abrasion and pathogens (Lehane and Billingsley 1996). Enzymes and nutrients move along the midgut through a countercurrent flow created in the space between the gut cells and the peritrophic matrix (Jiminez and Gilliam 1990).

The primary function of the midgut is the absorption of nutrients from the diet. This process is carried out by three functionally distinct classes of cell types found in the adult honey bee midgut: epithelial cells, endocrine cells, and stem cells (Wigglesworth 1965). The absorptive capacity of the intestine is due to the columnar epithelial cells, the principal cell type. These cells can be distinguished by their microvilli on the apical surface and the presence of prominent, centrally located nuclei (Lehane and Billingsley 1996). Residing among the columnar cells are two types of endocrine cells, a basal granular cell and a vesicular cell (Raes and Verbeke 1994). Both of these cells are of the open type, with a narrow extension that stretches to the lumen in order to release their contents along the basal surface. Both basal granular and vesicular endocrine cells are shed into the lumen with vesicles intact, indicating a possible exocrine function as well (Raes and Verbeke 1994).

The intestinal stem cells (ISCs) are the precursors of both midgut epithelial and endocrine cells (Raes et al. 1994). They are situated in regenerative crypts adjacent to the basement membrane and are not exposed to the lumen. These cells have been identified in insect species from the Collembola, Isoptera, Blattodea, Orthoptera, Diptera, Coleptera, Lepidoptera, and Hymenoptera (Corley and Lavin 2006). The role of ISCs in larval development has been well characterized in Lepidoptera, and replicative stem cells

have been identified in the midguts of larval and adult *Drosophila melanogaster* (Hartenstein and Jan 1992, Ohlstein and Spradling 2006), *Apis cerana* (Zhang et al. 2009) and *Apis mellifera* (Ward et al. 2008).

Given that the adult honey bee midgut is constantly undergoing apoptosis (Gregorc and Bowen 1997), stem cell differentiation must continue throughout the life of the bee in order to maintain functional intestinal tissue. This is especially significant when the rate of epithelial cell death is increased by stressors, such as exposure to pathogens (Gregorc and Bowen 2000) or pesticides (Gregorc et al. 2004, 2011). Research of ISC proliferation has shown that in adult honey bee workers, the proliferation of the ISCs declines with age and adapts to nutritional demand (Ward et al. 2008, Willard et al. 2011). Thus, the proliferative capacity of the ISCs may be a sensitive indicator of honey bee health and warrants further research.

Should proliferative capacity of the ISCs of the midgut be linked to honey bee health, it is essential that more work be done to characterize their cellular physiology. There are two approaches through which to investigate physiology—*in vivo* and *in vitro* techniques. *In vivo* techniques use a living insect, and are useful for understanding interactions between systems. However, *in vivo* techniques are limited in their ability to specifically understand how cells function in detail due to the lack of isolation. In contrast, *in vitro* studies provide a useful tool for such investigations through cell culture. *In vitro* studies also facilitate exploration of gene function and pathogenesis of intracellular parasites. Cell culture methods were promoted by the formulation of

solutions that keep cells alive outside of the host, and as close to *in vivo* conditions as possible.

Cell culture methods have given entomologists the capability to study individual cells, their development and differentiation, cytotoxicity, and gene expression (Morgan and Darling 1993). In fact, cell cultures have been developed from more than 100 insect species in order to meet these goals (Lynn 1996). Yet, there has been limited success in developing cell lines from *Apis mellifera*. Primary cultures have been developed from honey bee embryonic cells (Mitsuhashi 2001), neurons (Kreissl and Bicker 1992), and hemocytes (Beisser et al. 1990), and long-term cell lines have been established from undifferentiated embryonic honey bee cells (Bergem et al. 2006, Chen et al. 2010). However, lepidopteran larvae are currently the only insects with published protocols for midgut cell culture (Hakim et al. 2009, Castagnola et al. 2011). The development of techniques to culture cells from the honey bee midgut would provide new avenues with which to understand honey bee intestinal physiology and health at the cellular and molecular level while filling in gaps in current knowledge.

A cell line from honey bee ISCs would enable further studies to identify the factors that stimulate cell differentiation. In the prepupal insect, the larval midgut goes through extensive apoptosis and is rapidly replaced with adult midgut. This event is correlated with a rise in the steroid hormone 20-hydroxyecdysone (Lee et al. 2002). In *in vitro* studies of the ISCs of the larval caterpillar *Spodoptera littoralis*, addition of 20-hydroxyecdysone to culture media activated cell proliferation and promoted differentiation (Smagghe et al. 2005). This suggests that stem cell proliferation,

differentiation, and midgut apoptosis are under hormonal control. The role of 20-hydroxyecdysone in the honey bee midgut has not yet been evaluated. Characterizing the effects of this steroid hormone *in vitro* would add to the understanding of ISC proliferation and differentiation in the honey bee.

Specific aims

The first aim of this research project was to develop primary cell lines from the midgut of *Apis mellifera* and use these cultures to characterize the effects of 20-hydroxyecdysone on ISC proliferation and differentiation *in vitro*. This aim contributes to the basic characterization of the honey bee midgut and monitors the crucial function of replication in the ISCs. Based on previous findings in other cell types, 20-hydroxyecdysone is predicted to increase the replication rate of ISCs. In addition, primary cell cultures can be used as a study tool in several areas of applied or basic science. For example, this method could potentially be used as a starting point to develop screening techniques to assess the intestinal health of honey bee populations.

The second aim of this project was to establish a permanent cell line of stem cells from the midgut of *Apis mellifera*. Although known to be difficult, the development of a permanent cell line would represent a major investigative tool for honey bee research, enabling numerous molecular and biotechnology applications, including gene transformation, virology, immunology, developmental biology, and biochemistry. Thus, my project sought to develop methodology for basic and applied research that addresses

the current honey bee health crisis and allows for further insight into honey bee physiology.

CHAPTER II

METHODS AND MATERIALS

Materials

Apis mellifera honey bees were obtained from colonies in the research apiary maintained by the Rueppell lab in Greensboro, North Carolina. To obtain newly emerged and nurse bees, individual frames were removed from hives and placed in small frame boxes. Those frames were kept in an incubator. Newly emerged bees were carefully extracted directly from the cells from which they were emerging. Nurses were obtained by marking newly emerged bees with paint and allowing bees to mature in the incubator for two to seven days. Nurses were also identified in natural colonies as individuals that were observed with their heads down into brood cells. Foragers were captured at the honey bee station returning to their hives. This foraging activity usually starts when worker bees are between two and three weeks. Thus, the foragers were older than the nurses but their exact ages were unknown. Cultures from foragers were compared between those dissected immediately and those allowed to fly for one to two hours or overnight in a butterfly tent in order to clear the contents of their digestive systems

Dissection and dissociation

In order to determine the most effective strategy for creating primary cultures of intestinal cells, I tested three dissociation methods modified from existing protocols for

insect cell culture: enzymatic dissociation, tissue culture, and non-enzymatic dissociation. Each protocol was evaluated by determining the number and type of cells harvested and the percentage of those cells that were viable immediately after dissociation.

Method 1: Enzymatic dissociation

Dissection tools and dishes were soaked in 95% ethanol overnight prior to use. Honey bees collected for dissection were surface sterilized in 70% ethanol for two minutes and then their abdomens were severed from the thorax with microscissors. Abdomens were placed with the ventral surface exposed on a sterilized dissection dish filled with silicone elastomer. Pins were placed through the abdomen at the proximate and distal ends and sterilized microscissors used to carefully cut through the abdominal tergites. The interior of the abdomen was gently flushed with honey bee Ringer's solution (135 mM NaCl, 5 mM KCl, 180 mM sucrose, 20 mM HEPES, pH 6.7). The gut was then removed from the abdomen by making cuts at the proximal and distal ends. Malphigean tubules and other tissue debris was removed from the external surface of the gut. The peritrophic matrix and contents of the gut were removed with forceps. Finally, the gut was sliced longitudinally so that the interior surface was exposed. The gut was then placed into honey bee Ringer's solution in a sterile microcentrifuge tube and allowed to sit for two minutes. The Ringer's solution was then carefully removed and replaced with 1× trypsin-EDTA solution (Sigma T4174). The tissue was incubated in the trypsin solution for six minutes, after which the trypsin was removed and replaced with trypsin inhibitor (Sigma T-6522). The gut was incubated for five minutes, and then centrifuged

for one minute at 400xg. The supernatant was removed and replaced with various types of growth media. The gut was then manually dissociated by slowly pipetting up and down with a 1000uL pipette for approximately three minutes. Next, the tube was placed in a rack and the cells allowed to settle for three minutes. The supernatant was carefully removed and fresh media added to the tube. The suspended cells were then transferred to culture plates.

Method 2: Tissue culture

Dissection was completed as above. After the gut was removed from the abdomen, cleaned, and sliced longitudinally, it was gently rinsed with honey bee Ringer's and then placed whole into growth media in a culture plate.

Method 3: Cell filtration

Dissection tools and dishes were soaked in 95% ethanol overnight prior to use. Honey bees collected for dissection were surface sterilized in 70% ethanol for two minutes and then rinsed with sterile distilled water. The whole insects were then transferred using sterile forceps to a sterilized dissection dish filled with silicone elastomer and drenched in honey bee Ringer's with 0.001% sodium hypochlorite. The abdomens were separated from the thorax using sterile microscissors and transferred to a new dissection dish with fresh honey bee Ringer's solution and sodium hypochlorite solution and placed under a dissecting microscope (Figure 1).

The crop was visualized through the opening created at the proximal end of the abdomen when the thorax was removed. If the crop was not visible, the forceps could be used to carefully widen the opening. Gently, forceps were placed into the opening and used to grab the crop, which was then very slowly pulled through the opening. If done carefully, the crop would not break, even when full. Once the crop was exposed, the forceps were moved to the proventriculus, and the gut was pulled through the opening until fully exposed (Figure 2). Again, when done carefully, the gut would remain intact. This method leaves the majority of the Malphigian tubules within the abdomen, reducing the amount of non-gut tissue to be removed (Figure 3). Microscissors were then used to cut the gut at the distal end, separating it from the abdomen, and at the proximal end, to separate it from the crop. The guts were collected in fresh honey bee Ringer's and sodium hypochlorite solution and forceps used to remove any remaining Malphigian tubules or other tissues from the surface. The proximal and distal ends of the guts were removed, leaving only the midgut. The peritrophic matrix and its contents were removed with forceps, and the gut was again moved to fresh honey bee Ringer's and sodium hypochlorite solution. It was then sliced longitudinally to expose the interior surface.

Three to ten midguts were collected and placed in a 35-mm petri dish containing honey bee Ringer's with 0.001% sodium hypochlorite and soaked for two minutes (Figure 4). They were then moved to a new 35-mm dish containing honey bee Ringer's and soaked for two minutes. This was repeated twice. A sterile 40-um-pore-size filter basket (Fisher Scientific) was placed in a 35-mm petri dish and filled halfway with honey bee Ringer's solution. The midguts were placed in the basket, covered, and stirred for

fifteen minutes to one hour on an orbital stirrer set at sixty to ninety rotations per minute (Figure 4). After filtration, the filter basket was removed and a sterile pipette used to transfer all media and cells from the petri dish to sterile microcentrifuge tubes. The cells were centrifuged at 400xg for five minutes. Without disturbing the pellet, the solution was removed from the tubes and replaced with fresh honey bee Ringer's solution to wash by centrifugation. This was repeated for a total of three rinses. After the final rinse, the honey bee Ringer's solution was replaced with growth media and the suspended cells transferred to culture plates.

Cell viability assay

Cell viability was assessed with a Trypan blue vital stain. Trypan blue is a dye exclusion method of evaluating cell viability (Strober 2001). As the compound cannot be incorporated into cells with intact plasma membranes, cells that are stained blue when incubated in the dye are considered no longer viable. Living cells remain clear. To perform this assay, a sample of cells was placed in a siliconized microcentrifuge tube in a 1:1 solution of cells suspended in growth media and 0.4% Trypan blue. The cells were incubated in the Trypan blue solution for five minutes and then visualized under the microscope on a phase contrast hemocytometer. To use the hemocytometer, a 20 µl sample was loaded into the hemocytometer, and viable and non-viable cells were counted within nine randomly selected 1 mm^2 sections.

Optimizing cell culture conditions

All cultures were kept in an incubator at 27ºC. A 10 mL beaker containing distilled water was also kept in the incubator to provide humidification. Media were monitored daily for pH and bacterial growth. Cell adherence was evaluated by removing all media from the cultures and using a phase contrast microscope to determine the presence of cells in the culture dish and the media. Cells were tested for adherence to laminin, poly-L-lysine, Nunclon Opticell®, and non-coated dishes. Three complete cell culture media were compared for their effects on cell growth and vitality: Leibovitz L-15 media, Grace's insect media, and WH2 (Hunter 2010) media. Cell density was varied by increasing the number of guts used to make the culture from one to ten, and by varying the size of the well and amount of media in which the culture was placed. Volumes of 200 µl, 1 ml, and 2 ml of media were evaluated in parallel. Fat body extract (FBX) was tested in media at 0%, 10% and 20% concentrations. B27, a proprietary cell culture supplement utilized in the culture of *Apis mellifera* neurons, was also tested to see if there were any benefits to the ISC cultures. Penicillin/streptomycin and Amphotericin B were evaluated for their ability to control contamination and their toxicity to cells. In all trials, one-third of media volume was replaced with fresh media every 7 days in order to maintain pH, eliminate waste, and ensure appropriate nutrition.

Characterization of cell growth and proliferation *in vitro*

To quantify cell growth and compare media and storage conditions, growth curves were created for all experiments. Initially, cells were counted using a hemocytometer

every 24 hours, and the results graphed as cell number over time. However, as the experiment progressed, it became clear that cell densities after dissociation were not high enough for this to be an effective method of estimating cell numbers. Therefore, a phase contrast microscope was used instead for visualization and counting. Cell type and number were quantified in four consecutive fields of view across the diameter of the dish at 40x magnification. This was repeated for every culture every 48 hours. Vitality was also assessed using Trypan blue as described previously.

Characterization of the effects of 20-hydroxyecdysone on intestinal stem cell proliferation

My final experiment evaluated the differentiation of *Apis mellifera* intestinal stem cells in the presence of 20-hydroxyecdysone, a steroid hormone that triggers cell proliferation, differentiation, and death *in vivo* (Riddiford 1993). A concentration of 20 ng/µl (Hakim et al. 2009) was added to experimental cell culture dishes at the beginning of incubation. Cells were visualized every 48 hours and growth curves created as previously described.

Cellular proliferation assay

Previous research in our lab identified the intestinal stem cells as the only proliferative cells in the adult honey bee (Ward et al. 2008), using immunohistochemical labeling with 5-bromo-2'-deoxyuridine (BrdU). BrdU is a thymidine analogue that is incorporated into newly synthesized DNA (Shermoen 2000). To measure the presence

and timing of cell proliferation, samples of cultures were taken every 48 hours. Each sample was removed from the culture dish with a sterile pipette and placed in the wells of a non-coated 96-well plate. BrdU (Sigma) dissolved in S2 cell media (Sigma) was added directly to the medium of the sample culture to a final concentration of 1 mg/ml (Hakim et al. 2009). The plates were then sealed with Parafilm™ and placed in the incubator overnight.

After 20-24 hours, the samples were removed from the well plate and pipetted in 50uL aliquots onto SuperFrost Plus glass slides (Fisher). The slides were placed on a slide warmer at 50ºC for one hour to dry. They were then fixed in 70% ethanol for ten minutes. Next, the slides were rinsed in 1× PBS (137 mM NaCl, 2.68 mM KCl, 10.14 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.4) for five minutes and then blocked in a blocking solution of 0.001% Tween-20 (Fisher Scientific), 1% goat serum (Biomedia), and a small pinch of non-fat bovine dried milk (Sigma) in 1× PBS for five minutes. After blocking, the slides were submerged in 2N HCl for twenty minutes to denature the DNA. They were then rinsed in 1×PBS for five minutes three times. Anti-BrdU mouse anitbody (Phoenix Flow Systems) was prepared at a 1:100 dilution in the blocking solution of 0.001% Tween-20, 1% goat serum (Biomedia), and a small pinch of non-fat bovine dried milk (Sigma) in 1× PBS. The slides were placed horizontally in a humid chamber and the sample covered with the primary antibody solution and a "coverslip" of Parafilm™. They were then left to incubate overnight for 20-24 hours at 4ºC.

Slides were removed from the humid chamber and rinsed in 1× PBS with 0.05% Triton X-100 (PBST) for five minutes three times. Goat anti-mouse peroxidase

conjugated secondary antibody (Jackson ImmunoResearch Laboratories, Inc.) was diluted 1:40 in PBST and incubated horizontally in a humid chamber with a Parafilm™ "coverslip" for two hours at room temperature. Next, the slides were rinsed three times in PBST for ten minutes, and in 1× PBS twice for ten minutes. The slides were incubated in activated diaminobenzidine (Sigma) six to eight minutes until a color change was observed, and then immediately rinsed for one minute in distilled water three times. Finally, the slides were dehydrated in a series of increasing ethanol concentrations (50%, 70%, 95%, 100%, 100%) for five minutes each. Coverslips were mounted to the slides with Permount (Fisher) and allowed to dry horizontally. Negative controls did not have BrdU added to the media, or were not incubated in primary antibody. The slides were viewed with a 40X objective on a light microscope. Each slide was visually scanned in its entirety for the presence of cells with labeled nuclei.

Analyses

Most of my efforts yielded descriptive results that did not require further quantitative analyses. For the final experiment, I evaluated the impact of age and exposure to 20-hydroxyecdysone on lifespan with a Cox regression survival analysis and two-factorial ANOVA. ANCOVA was used to determine whether there was a significant difference between the average number of stem and differentiated cells over time between cultures from pre-eclosion pharate adults, newly emerged, nurse, and forager donors.

CHAPTER III

RESULTS

Establishment of primary cell cultures

Tissue culture

Ten tissue cultures from both newly emerged and forager workers were established. All of these cultures resulted in severely contaminated cultures despite rinsing the tissue, sterile technique, and the addition of antimicrobials to the media. Allowing foragers to fly in a cage without food overnight in order to empty their gut prior to dissection did not reduce contamination. Further, the cells were non-adherent to dishes and stem cells could not be visualized or separated from other cells. Thus, it was concluded that tissue culture was an ineffective method for this project.

To directly compare the two media used until this point, I set up tissue cultures of three newly emerged and three foragers (starved overnight). Each bee gut was divided in half after dissection. Half the gut was placed in L-15 media with B27 and penicillin/streptomycin and half in Grace's media with 20% FBX and penicillin/streptomycin. There was no noticeable difference between the culture density, shape or viability of the cells from the newly emerged workers after 24 hours. The cells from the foragers did not survive in culture.

Cell dissociation and sterilization

Cell culture, following enzymatic dissociation has been successfully utilized to culture *Apis mellifera* neurons (Fahrbach et al. personal communication). This protocol was followed, substituting the midgut for mushroom bodies as initial material. Newly emerged workers were used as the tissue source, and the cells were cultured in L15 media supplemented with B27 and 10% penicillin/streptomycin. While small, round cells were visible on the hemocytometer, testing with Trypan blue indicated that the cells were not viable. Cells were harvested in 6 attempts using this method without improvement. The method was then modified to decrease the amount of exposure to trypsin enzyme. Cells were allowed to dissociate for 1 minute, 2 minutes, 4 minutes, 6 minutes, and 8 minutes before the enzyme was inactivated. While dissociation for two minutes required more manual dissociation with the pipette, this set of samples also produced a greater number of rounded cells of a more regular shape than those exposed to four, six, or eight minutes of dissociation. However, the cells harvested were not viable immediately after dissociation, and there were no cells found in the culture 24 hours later. Thus enzymatic dissociation was determined to be an ineffective method for harvesting stem cells from the midgut.

At this point, I switched my approach to developing a technique utilizing non-enzymatic tissue dissociation by modifying the technique from Hakim et al. (2009) utilizing an orbital stirrer and tissue basket to separate cells after dissection. Initially, I tested Grace's insect medium with varying levels of FBX, 50 units/mL penicillin/streptomycin, and 0.25 µg/mL Amphotericin B, as called for in the Hakim

protocol. Allowing the tissue to spin in the orbital stirrer for 120 minutes at 1.5 revolutions/second resulted in a small number of cells being released into the media. In the first round working with this technique, I cultured 21 newly emerged workers, 6 nurses, and 10 foragers with 10% FBX added to the media, and 10 newly emerged workers and 16 foragers in 20% FBX media. There was significant contamination within 48 hours in all of these cultures.

I increased the amount of penicillin/streptomycin to 100 units/mL, but this did not prevent contamination within three days. For my next trial, I increased the time in which the dissected midguts were rinsed. I dissected the midguts from 20 newly emerged workers and soaked them in 0.001% sodium hypochlorite in honey bee Ringer's solution for 10 minutes followed by two two-minute rinses. In addition, I prepared two negative control plates each containing only Grace's media or Ringer's solution to ensure that my media were not contaminated. After 6 days, the media remained uncontaminated, thus I concluded that this was not the source of the contamination in my cultures.

The cultures from the newly emerged workers rinsed for 14 total minutes remained uncontaminated for 21 days. However, the number of cells in these cultures was too low to perform viability or proliferation assays. I continued to attempt to improve my technique by culturing 15 newly emerged workers but allowing them to remain on the stirrer for 180 minutes. The number of cells acquired was greater, but these cultures were severely contaminated within 24 hours and were discarded.

Growth conditions

Adherence of cells was tested on poly-D lysine-coated cover slips, laminin-coated glass cover slips, Nunclon-coated cell culture dishes, and non-coated dishes. To test adherence to the coated cover slips, cells were allowed to settle for 5 minutes, 45 minutes, and 1 hour. Additionally, the media washed from these surfaces was saved and visualized under the microscope to evaluate for non-adherent cells. No cells were found to adhere to the cover slips. However, cells were present in the media washed from the coverslips and culture dishes after the adherence time trials. An additional trial of Nunclon-coated dishes allowed to incubate for 1 hour yielded the same results—the cells were present in media but did not adhere to the Nunclon surface. Cells in L15 media in Nunclon dishes did not survive for 24 hours in the incubator (n=10). In sum, I was unable to identify any surface to which the cells adhered (Table 1). Future efforts were therefore geared towards maintaining free-floating cells in media.

At this point I continued the attempt to compare varying levels of FBX and combat my contamination problem. I modified my protocol to include four 0.001% sodium hypochlorite rinses of two minutes each and reduced the time on the orbital stirrer to 60 minutes. In order to reduce variability between cultures, rather than using different bees for each media trial, I divided my cultures from the same bees into wells with 0% FBX, 10% FBX, and 20% FBX. The numbers of cells was still very small. Further, there appeared to be clusters of cells in the dishes, making counting them with the hemocytometer an inaccurate representation of the total number of cells present. I therefore decided to utilize an inverted scope to visualize these cells, which required

transporting the cells to Wake Forest University. I placed the Parafilm™-wrapped culture dishes in a Styrofoam cooler for transport. Any exposure to air can potentially lead to culture contamination, but this technique allowed the cultures to be counted without opening the dishes, reducing this risk. This experiment was successful in that I was able to visualize and count cells more accurately than with the hemocytometer and only the 20% FBX cultures were contaminated. The numbers of cells was not significantly different between the varying levels of FBX and all cultures showed a decrease in cell numbers within the first 48 hours of culture (Table 2, Figure 5).

At this point, I was still struggling with contamination in some of my cultures. Despite repeatedly cleaning the cell culture lab and utilizing sterile techniques, my FBX cultures were more consistently contaminated than those without. As the FBX appeared to provide no benefit and was resource-intensive to obtain, I decided to no longer include FBX in further experiments.

I expanded the experiment to include cultures in L-15 and WH2 media for comparison. Upon observation, there was no difference in the numbers of cells in any media. However, the cells in the WH2 media more often appeared in clusters (Figure 6). When evaluated on the inverted microscope, I was able to visualize two distinct cell types in the WH2 cultures, one a stem-cell like round cell, and the other an oblong cell, potentially differentiated. I therefore decided to only utilize WH2 media in the remainder of my experiments in order to simplify comparisons.

I attempted to increase the number of cells obtained by reevaluating the enzymatic dissociation protocol in combination with the filter basket, but there was so much debris

in the culture that I was unable to visualize the cells, thus this method was not explored any further.

Cellular proliferation assay

Once I was sure I could successfully isolate cells in cultures utilizing the cell filtration method, and keep over 50% of my cultures uncontaminated for at least one week I began the next stage of the experiment, which was to utilize BrdU staining to determine if the cells in my cultures were proliferative. After multiple trials, I was able to successfully demonstrate proliferation in a 7-day-old culture and finalized my staining procedure. Samples were obtained every other day from each control and 20-hydroxyecdysone treated culture and stained immediately. Stained cells were observed in cultures from each age and treatment with the exception of foragers, but the number of positive cells was extremely small. The time at which proliferation was observed was also highly variable. (Table 6) I did not systematically evaluate the effects of dissociation and sterilization methods or growth media on cell proliferation.

Effects of age and 20-hydroxyecdysone

Once I had established cell culture, measurement, and BrdU staining procedures that could be consistently replicated, I entered the final phase of the experiment, in which I directly compared cultures from pre-eclosion pharate adults, newly emerged workers, nurses, and foragers with and without ecdysone for the number and morphological characteristics of cells that grew and the longevity of the culture. I also performed BrdU

staining on samples from the cultures throughout the experiment to determine whether the cells were proliferative throughout the course of the experiment. These cultures were established over a shorter period of time (all cultures were started within a two week period), allowing for more direct comparison than in my previous experiments.

The resulting cultures were viable for up to 15 days (Figure 7). Cox regression analysis showed that age of donor had no significant effect on the longevity of cultures ($p=0.75$). This was confirmed by two-factorial ANOVA ($F_{(2,54)} =1.20$, $p=0.32$). The addition of 20-hydroxyecdysone did not significantly alter the longevity of the cultures established from individuals at any age (Figure 8), as confirmed by Cox regression analysis ($p=0.95$) and two-factorial ANOVA ($F_{(2,54)} =0.14$, $p=0.71$). There was also no significant interaction between the age and treatment with 20-hydroxyecdysone ($F=0.19$, $p=0.90$).

Both the control cultures and 20-hydroxyecdysone treated cultures showed potential cell differentiation in pre-eclosion pharate adults (Table 3, Figure 9), newly emerged (Table 4, Figure 10), nurse (Table 5, Figure 11), and forager cultures (Table 7, Figure 12). I classified cells as potential stem cells, goblet cells, or columnar cells based on their appearance in culture using criteria from Hakim et al. 2009. Cells that were spheroid were classified as stem cells, those that were rectangular in shape with large, central nuclei or gourd-like were classified as differentiated cells for the purpose of analysis. Morphologic characteristics alone are not sufficient to definitely call these cells differentiated, and further analysis is required, however the morphology is suggestive of

three distinct cell types in these cultures. For the purposes of this analysis, I will refer to these three distinct morphologies by as stem cells, columnar cells, and goblet cells.

ANCOVA analysis was completed to determine whether there was a difference in the number of cells of each type by age. I calculated a ratio of the average number of differentiated cells to stem cells for each age group. ANCOVA indicated the ratio was significantly different between pre-eclosion pharate adults and workers ($F=10.33$, $p=0.00015$). In order to determine whether this was a factor of the stem cells, differentiated cells, or both, I used ANCOVA to analyze each cell type separately. The average number of stem cells over the life of the culture was significantly different between pre-eclosion pharate adults and workers ($F=12.95$, $p=0.000031$), but the average number of differentiated cells was not ($F=4.06$, $p=0.018$).

BrdU staining confirmed stem cell proliferation in control and 20-hydroxyecdysone-treated cultures from pre-eclosion pharate adults, newly emerged workers, and nurses but not from foragers under either condition (Table 6). Overall, there was very little proliferation, especially as compared to differentiation. Due to this result, I was unable to achieve my second experimental aim, the establishment of long-term or permanent cell cultures. While some of my cultures were proliferative, I was never able to recover stem cells after the second passage.

CHAPTER IV

DISCUSSION

The establishment of new cell cultures is a challenging process, and often more of an art than a hard science. Thus, I had to try a large number of different protocols in order to determine what did and didn't work. At the end of this project, I had determined a protocol to successfully culture and maintain intestinal stem cells from the midgut of the honey bee. These cultures could be maintained for up to 15 days. Additionally, I developed a technique for performing BrdU staining on these cells in order to determine whether or not the cells are proliferative. This is the first successful culture of these cells from adults of the domestic honey bee, *Apis mellifera*. In all, I initiated 590 cultures under 46 different conditions (including variations in protocol at all stages) in the course of this experiment. The following is a summary and discussion of a number of observations made during this process.

The number of cells extracted at the beginning of the procedure seems to affect the density of the resulting culture. Obtaining too few cells reduces the longevity of the culture, while too many cells increases the risk of contamination. The ideal protocol involved dissociation of 3-4 guts/well, spun on the orbital stirrer for 60 minutes. However, it is also possible that the density of the cell cultures was affected by the health of the bee, which was beyond the scope of this experiment and should be considered.

Under the microscope, a clear, observable difference between healthy and unhealthy/dying stem cells can be observed (Figure 13). The unhealthy cells had an irregular border, and appeared somewhat bubbly. They were also optically dense, while healthy cells were clearer. Frequently, a healthy cell was found next to an unhealthy or dead cell. One possible explanation for this is that unsuccessful cell division had recently occurred.

All cells floated above the bottom of the culture dishes and did not attach, regardless of the coating on the dish. Differentiated cells types floated higher in the media than the round stem cells. I found optically clear, uncoated 6-well dishes to be the most effective for these cultures. The cultures should be staggered, with an empty well between each culture to reduce the risk of contamination.

A relatively new culture medium, WH2, was found to be comparable to L-15 and Grace's insect media for growth of cultures. WH2 has been utilized for the development of cell cultures from larval and pre-eclosion pharate adults honey bee donors (Hunter 2010) but was found in this study to be adequate for all ages. Previous honey bee cultures have required supplemented media for long-term maintenance (Bergem et al. 2006). My results indicate that the WH2 media is an acceptable substitute, and in fact may be preferable to other media as it is inexpensive and easy to formulate.

My observations were similar to results that were obtained using similar techniques with the midgut of *Manduca sexta* and *Heliothis virescens*: the cells were non-adherent, and two distinct cell types were observed (Hakim et al. 2009, Castagnola et al. 2011). This is quite different from embryonic and neuronal cell cultures from *Apis mellifera*,

which are adherent to culture surfaces. The result is also unexpected because *in vivo* ISCs reside along the basement membrane of the intestine and are thus adherent. However, as the stem cell differentiates *in vivo,* it dissociates from the basement membrane in order to be exposed to the intestinal lumen. Therefore, it is possible that the stem cell cultures would be adherent if differentiation could be suppressed in these cultures. Adherent cultures would be useful for practical purposes, allowing for easier visual inspection by microscope, increased ease and accuracy of cell counts, and permitting individual cells to be tracked through time. Suppression of differentiation would allow exploration of stem cell physiology without confounding factors created by differentiated cells. Regardless, the similarity in results to that of lepidopteran ISC cultures indicates thus this technique could potentially be utilized to culture the adult midgut from additional insect species.

The stem cells obtained in culture varied widely in size but the columnar cells did not (Figures 15, 16). The variation in size of stem cells is consistent with observations of lepidopteran stem cell cultures (Castagnola et al. 2011) and possibly implies that the stem cells in culture were in a variety of cell cycle stages. Additionally, I observed cells that were more oblong in appearance with one long, skinny end consistent with goblet cells (Figure 16). These cells appeared on day 5-6 of the culture and were very few in number. This indicates that cells differentiated *in vitro* according to their natural developmental fate. Differentiation has also been observed in lepidopteran midgut cultures (Hakim et al. 2009, Castagnola et al. 2011). This result suggests that the conditions in the cell cultures are representative of the *in vivo* intestinal environment, and indicates that these cultures are appropriate for use as a model of midgut molecular and cellular physiology.

There was no significant difference in the type or longevity of cells obtained from culture of pre-eclosion pharate adults, newly emerged, nurse, or foragers. On account of these results, the techniques described here are suitable for culture of any aged honey bee. It should be noted that contamination rates were elevated in nurses and foragers. I attempted to reduce contamination rates by allowing emerged workers to defecate before dissection, but this method did not reduce contamination. Contamination was highly variable and it is possible that contamination is an indicator of the health of the gut. Therefore, it would be interesting to classify the bacterial or fungal contamination of cultures compared with the normal flora present in the bee gut (Martinson et al. 2011) and determine whether this technique could be used as an indicator of midgut health.

While there was no significant effect of age of donor on culture lifespan, the behavior of the stem cells in culture was impacted by age. The number of stem cells was significantly different in cultures from pre-eclosion pharate adults bees as compared to that of workers while the average number of differentiated cells was not significantly different across age groups. This result implies that the behavior of the stem cells in culture was impacted by the age of the donor, while differentiation was independent of age. Notably, no proliferation was observed in cultures established from forager donors. At this time, there are no studies found in the literature comparing insect midgut cultures from multiple ages. Yet, comparison of ISC proliferation in bees across ages *in vivo* has shown the number of proliferative cells to decline with age (Ward et al. 2009). My *in vitro* results support the idea that ISCs lose their proliferative capacity with age. Thus, the protocol for primary culture developed here could be utilized to test environmental

factors *in vitro* such as nutrition, pathogens, or toxic exposure to further elucidate their impact on intestinal stem cell replication and ultimately, intestinal health.

The steroid hormone 20-hydroxyecdysone had no effect on the rate of differentiation or the longevity of the cultures. In experimental *S. littoralis* and *M. sexta* cultures, 20-hydroxyecdysone leads to increased differentiation of ISCs into columnar and goblet cells (Smagghe et al. 2005, Hakim et al. 2005). 20-hydroxyecdysone has also been shown to inhibit mitotic activity in Kenyon precursor cells from *Apis mellifera* (Malun et al. 2003), but there was no difference in proliferation between control and treated cultures in this experiment. It can therefore be concluded that *in vitro* 20-hydroxyecdysone has no effect on proliferation or differentiation of ISCs in the honey bee midgut at the delivered concentrations. In *S. littoralis* cultures, the effect of the hormone was found to be dose dependent; it is possible that the dosage applied in this experiment, while appropriate for lepidopteran cells, was inappropriate for these cells. Alternatively, the honey bee midgut cells may not have the appropriate nuclear receptor for the hormone. If this is the case here, it could be confirmed through 9B9 antibody staining procedures or a measure of gene expression through qRT-PCR (Smagghe et al. 2001).

A second goal of this experiment was to create a permanent honey bee cell line. This was not achieved using the evaluated techniques. The biggest obstacle was that the cultures were short-lived, with a maximum longevity of 15 days. Additionally, the cultures were of low cell density and mixed cell types. To attempt to establish a

permanent cell line, more work is needed to improve cell proliferation and viability *in vitro*. The methods described here will provide a starting point for such an endeavor.

It is possible that the longevity and proliferation achieved here can be improved upon, as lepidopteran midgut cultures can survive for several months (Hakim et al. 2009). However, embryonic honey bee cells in culture differentiate rather than proliferate when over 36 days of age (Bergem et al. 2006). Over the life of the cultures observed in my study, the number of stem cells tended to decrease while the number of differentiated cells was more likely to increase. This suggests that the stem cells in the cultures were differentiating in culture rather than creating new stem cells, even though some proliferation was measured and potentially replicating stem cells were observed (Figure 17). One possible explanation for this is that the stem cells that stained positively with BrdU were already mitotically active when they were harvested. Alternatively, this could be explained by a low rate of cell replication coupled with a high rate of differentiation. The ISCs would need to be stimulated to proliferate in culture before establishing a permanent cell line could be attempted. Thus, long-term cultures are not possible utilizing this technique as described.

The nature of this project made it difficult to directly compare cell culture conditions until a methodology was established. This is the most significant weakness of these experiments. In addition, even when cell culture was successful, there were still struggles with contamination and very few of the cultures survived to the end of the experiment. Therefore, these methods can best serve as guidelines for future work.

This culture technique may be utilized for future *in vitro* studies of the honey bee midgut, but also may prove to be useful as an indicator of the health of the digestive system in bees in a given population. When I accidentally used bees that were infected with protozoans during this experiment, parasites were easily seen as soon as the culture was established. This result could easily be exploited for health monitoring purposes. Further work comparing the cultures of healthy and unhealthy bees would clarify whether parameters such as longevity of the cell culture or contamination are indicative of the condition of the midgut. If there is a difference, then this technique could potentially be used to culture sentinel bees from a hive to determine the health status of the population.

In sum, my experiments resulted in the development of the first technique for primary cultures of ISCs from the honey bee. Although problematic, these cultures may provide a mechanism for *in vitro* study of the molecular and cellular physiology of the honey bee midgut, allowing for future applications of the honey bee as a model research organism and further investigation into the role of the midgut in honey bee health.

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APPENDIX A

TABLES AND FIGURES

Table 1. Adherence and cell viability as related to surface coating and growth media. Cells cultured on poly-D lysine, laminin, and nunclon were not viable and cells did not adhere to any dish coatings. Cells were viable in all tested media. Multiple morphologies were observed in cultures with Grace's media and WH2 media. Y= yes, N = no.

	Coating				**Media**			**Results**		
Experimental condition	Poly-D lysine	Laminin	Nunclon	None	L15	Grace's	WH2	Adherence	Viability	Multiple cell types
1	Y				Y			N	N	N
2		Y			Y			N	N	N
3			Y		Y			N	N	N
4				Y	Y			N	Y	N
5				Y		Y		N	Y	Y
6				Y			Y	N	Y	Y

Table 2. Comparison of number of stem cells observed in cultures with 0%, 10% and 20% FBX. Cultures were grown in Grace's media with 1 ug/ml penicillin/streptomycin and 0.25 µg/ml Amphotericin B. Donor honey bees were all less than one week of age, n = 8 per group. Averages are given with the total range in brackets.

	0% FBX	**10% FBX**	**20% FBX**
Day 0	97.1 (34-145)	141.1 (72-328)	125.4 (61-183)
Day 1	57.3 (0-119)	91.4 (71-195)	67.4 (0-131)
Day 2	65.9 (0-141)	63.1 (52-146)	61.8 (0-130)
Day 3	28.5 (0-89)	37.4 (0-135)	46.9 (0-145)
Day 4	62.4 (0-265)	73.3 (0-312)	58.0 (0-199)
Day 5	86.9 (0-284)	91 (0-220)	67.0 (0-96)
Day 6	16.8 (0-83)	37.5 (0-233)	7.5 (0-60)

Table 3. Number of stem and differentiated cells observed in cultures from pre-eclosion pharate adults honey bee donors with and without the addition of 2 µg/ml 20-hydroxyecdysone (20-HE). Cultures were grown in WH2 media with 1 µg/ml penicillin/streptomycin and 0.25 µg/ml Amphotericin B, n = 6. Averages are given with the total range in brackets.

	Control		**20HE**	
	Stem	**Differentiated**	**Stem**	**Differentiated**
Day 1	5.1 (3-7)	0.67 (0-2)	6.7 (4-7)	0.16 (0-1)
Day 3	6.3 (2-10)	1.8 (0-3)	6.3 (4-9)	0.33 (0-1)
Day 5	3.8 (0-9)	0.50 (0-2)	6.0 (4-10)	1.5 (0-4)
Day 7	5.7 (3-11)	2.8 (0-5)	4.8 (2-7)	2.1 (1-6)
Day 9	4.3 (2-7)	2.5 (1-6)	3.2 (0-5)	2.0 (0-5)
Day 11	4.7 (0-6)	1.3 (1-3)	4.5 (0-8)	0.83 (0-2)

Table 4. Number of stem and differentiated cells observed in cultures from newly emerged honey bee donors with and without the addition of 2 µg/ml 20-hydroxyecdysone (20-HE). Cultures were grown in WH2 media with 1 µg/ml penicillin/streptomycin and 0.25 µg/ml Amphotericin B, n = 12. Averages are given with the total range in brackets.

	Control		**20HE**	
	Stem	**Differentiated**	**Stem**	**Differentiated**
Day 1	8.0 (5-17)	0.50 (0-2)	8.3 (3-16)	1.3 (0-7)
Day 2	2.6 (0-6)	0 (0)	1.3 (0-6)	0 (0)
Day 3	2.5 (0-6)	0.58 (0-2)	2.7 (0-10)	1.5 (0-5)
Day 5	1.8 (0-5)	2.3 (0-7)	3.6 (0-8)	2.2 (0-5)
Day 7	5.3 (0-12)	1 (0-4)	2 (0-6)	0.83 (0-3)
Day 9	1.2 (0-5)	0.75 (0-5)	1.3 (0-4)	1.4 (0-5)
Day 11	1.5 (0-5)	0.92 (0-5)	1.4 (0-5)	0.92 (0-4)
Day 13	0.58 (0-3)	0.75 (0-4)	0.5 (0-3)	1.1 (0-5)
Day 15	0.083 (0-1)	0.42 (0-5)	0.33 (0-4)	0.083 (0-1)

Table 5. Number of stem and differentiated cells observed in cultures from nurse honey bee donors with and without the addition of 2 µg/ml 20-hydroxyecdysone (20-HE). Cultures were grown in WH2 media with 1 µg/ml penicillin/streptomycin and 0.25 µg/ml Amphotericin B, n = 14. Averages are given with the total range in brackets.

	Control		**20HE**	
	Stem	**Differentiated**	**Stem**	**Differentiated**
Day 1	5.9 (3-10)	1.8 (0-5)	5.5 (0-8)	2.3 (0-6)
Day 3	3 (0-5)	2.1 (0-5)	1.8 (0-7)	2.1 (0-4)
Day 5	1.6 (0-7)	1.3 (0-5)	1.1 (0-5)	0.93 (0-5)
Day 7	1.4 (0-50	1.5 (0-9)	1.1 (0-5)	1.4 (0-7)
Day 9	1.1 (0-4)	1.7 (0-7)	0.64 (0-3)	2.0 (0-10)
Day 11	0.87 (0-3)	1.4 (0-7)	0.93 (0-4)	0.8 (0-3)
Day 13	0.86 (0-4)	1.1 (0-6)	0.64 (0-2)	1.6 (0-5)

Table 6. Number of cultures positive for cellular proliferation by BrdU staining by age group and treatment.

	Day 2	Day 4	Day 6	Day 8	Day 10	Day 12	Day 14
Pre-eclosion pharate adult control	0	0	0	1	0	0	0
Pre-eclosion pharate adult 20HE	0	1	0	0	0	0	0
Newly emerged control	2	2	0	0	0	0	0
Newly emerged 20HE	0	0	1	0	0	1	0
Nurse control	0	1	1	1	0	1	0
Nurse 20HE	0	0	1	0	0	0	0
Forager control	0	0	0	0	0	0	0
Forager 20HE	0	0	0	0	0	0	0

Table 7. Number of stem and differentiated cells observed in cultures from forager honey bee donors with and without the addition of 2 µg/ml 20-hydroxyecdysone (20-HE). Cultures were grown in WH2 media with 1 µg/ml penicillin/streptomycin and 0.25 µg/ml Amphotericin B, n = 9. Averages are given with the total range in brackets.

	Control		**20HE**	
	Stem	**Differentiated**	**Stem**	**Differentiated**
Day 1	1.0 (0-7)	0.64 (0-5)	1.8 (0-14)	0.50 (0-3)
Day 3	1.0 (0-10)	0.36 (0-3)	1.1 (0-3)	0.57 (0-2)
Day 5	0.79 (0-5)	1.1 (0-5)	1.9 (0-7)	1.3 (0-8)
Day 7	0.43 (0-4)	0.93 (0-6)	1.2 (0-8)	0.93 (0-4)
Day 9	0.79 (0-4)	0.57 (0-3)	0.43 (0-4)	0.79 (0-7)
Day 11	0.57 (0-6)	0.71 (0-6)	0.29 (0-2)	0.43 (0-3)
Day 13	0.36 (0-3)	0.43 (0-4)	0.29 (0-3)	0.5 (0-5)
Day 15	-	-	0.21 (0-3)	0.36 (0-5)

Figure 1. Removal of the abdomen from the thorax. The abdomen was separated from the thorax of the honey bee and placed in fresh honey bee Ringer's with 0.001% sodium hypochlorite for dissection.

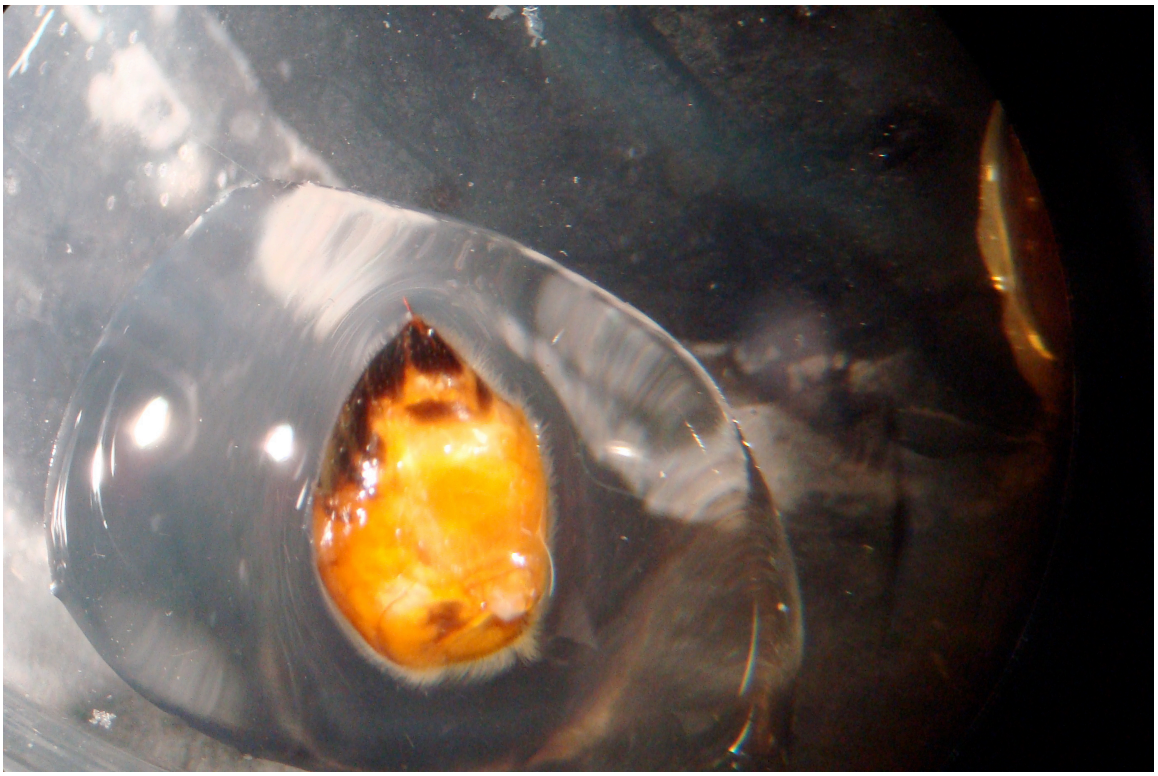

Figure 2. Removal of the gut from the abdomen. The entire gut was removed carefully from the opening in the proximal end of the abdomen. The crop has been removed from this specimen for better visualization, but should be extracted prior to the gut.

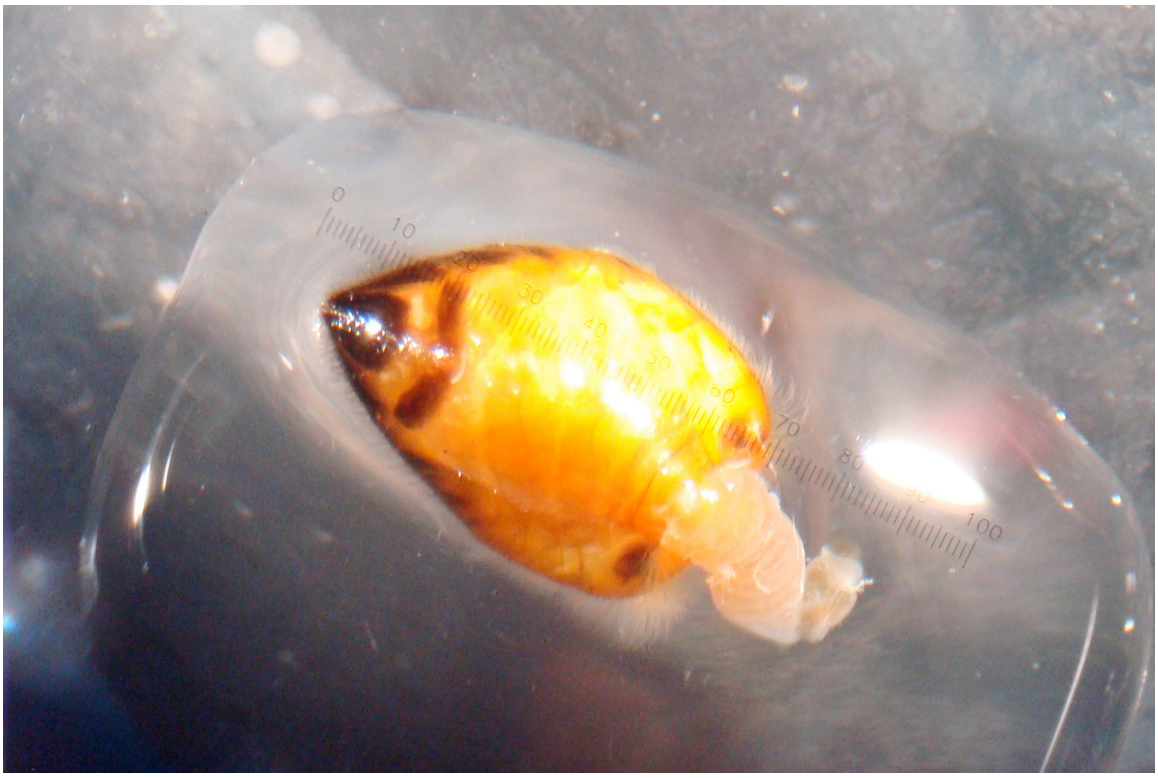

Figure 3. The extracted gut. When the gut is extracted intact through a small opening in the proximal end of the abdomen, the majority of Malphigian tubules remain within the abdomen, reducing the amount of non-gut tissue to be removed prior to dissociation.

Figure 4. Dissociation on the orbital stirrer. Dissected midguts in media were placed in filter baskets within petri dishes on the orbital stirrer for dissociation. A clean, plastic container was placed over the dishes to reduce risk of contamination.

Figure 5. Comparison of average number of stem cells observed in cultures with 0%, 10% and 20% FBX over 6 days. Cultures were grown in Grace's media with 1 µg/ml penicillin/streptomycin and 0.25 µg/ml Amphotericin B. Donor honey bees were all less than one week of age, n = 8 per group. There was no observable difference in the average number of cells or longevity between the cultures.

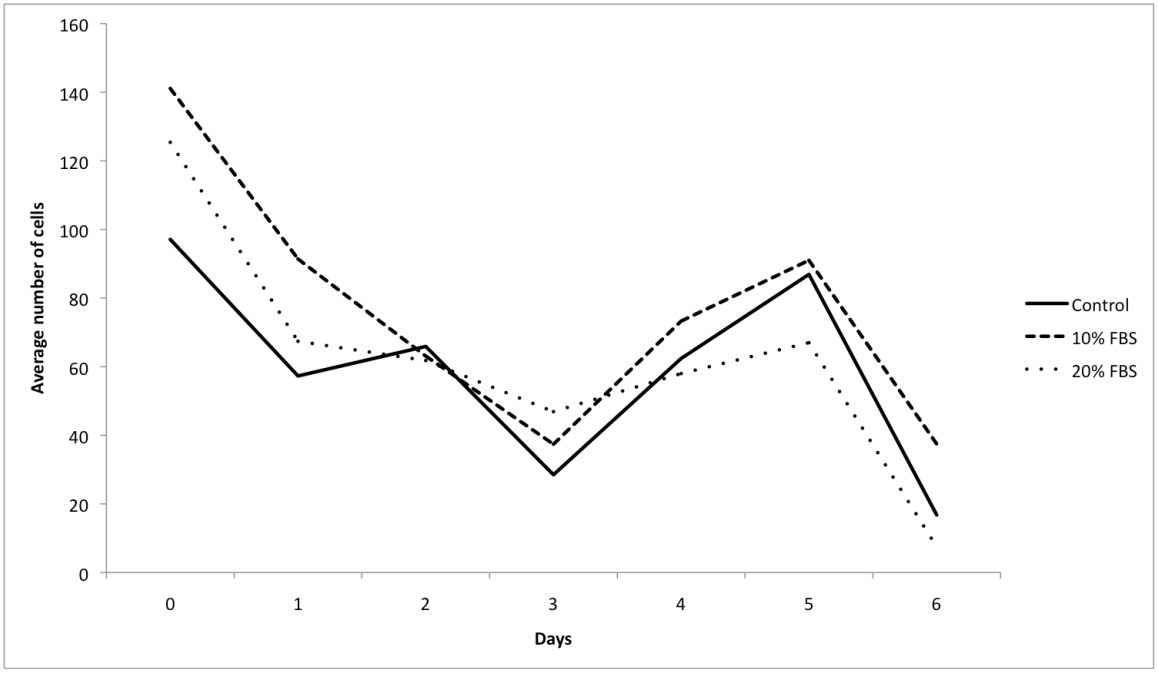

Figure 6. Clusters of cells in culture. Cells in culture were often found in clusters.

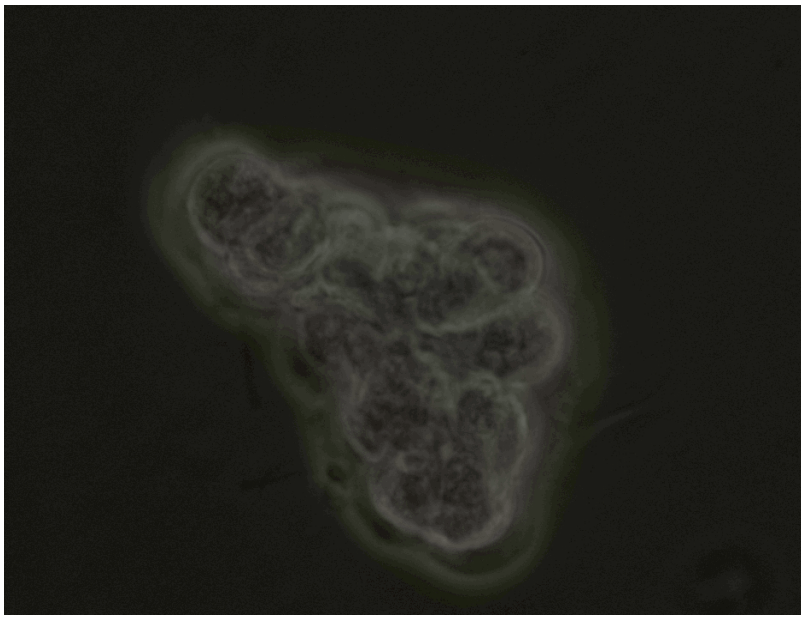

Figure 7. Probability of survival was not a function of donor honey bee age.

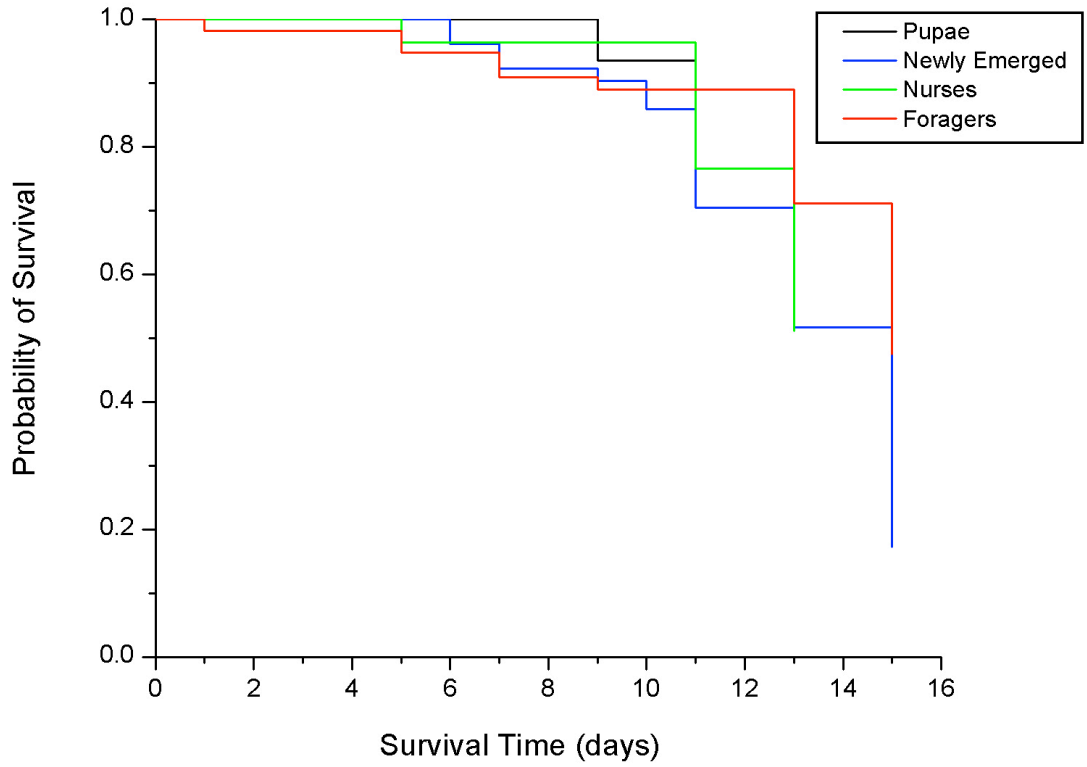

Figure 8. Probability of survival of control cultures was not significantly different than that of cultures treated with 20-hydroxyecdysone.

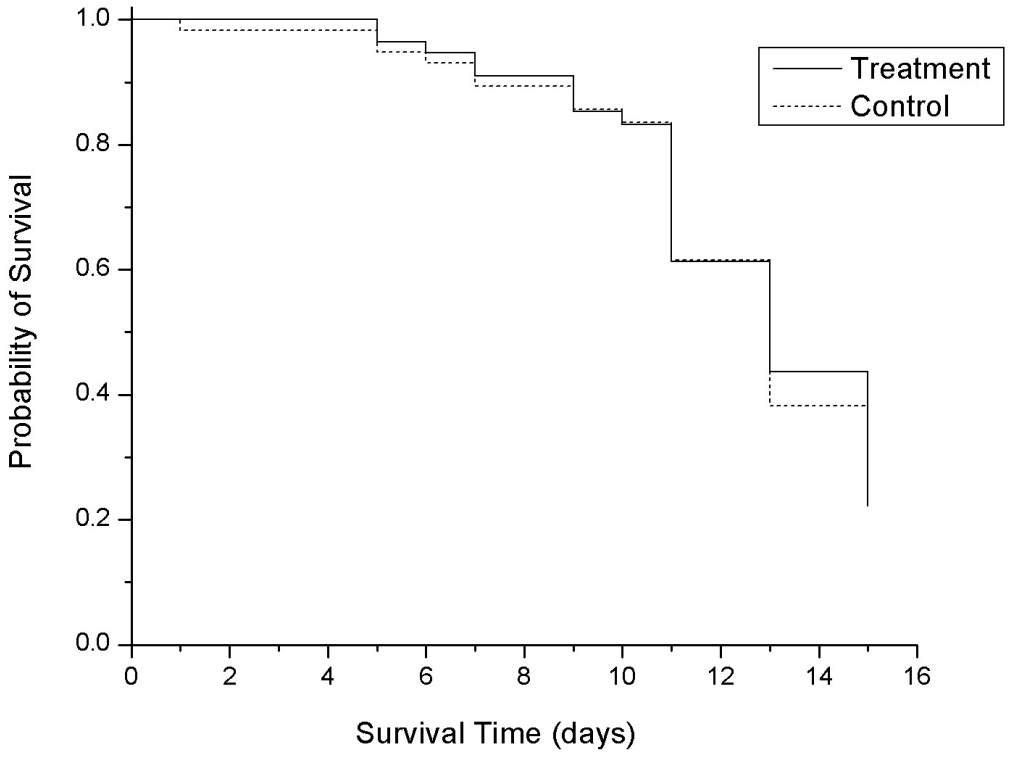

Figure 9. Average number of stem and differentiated cells observed over the lifespan of cultures from pre-eclosion pharate adults honey bee donors with and without the addition of 2 µg/ml 20-hydroxyecdysone. Cultures were grown in WH2 media with 1 µg/ml penicillin/streptomycin and 0.25 µg/ml Amphotericin B, n = 6. The average number of stem cells was higher than that of differentiated cells. Addition of 20HE did not impact cell numbers or morphologies.

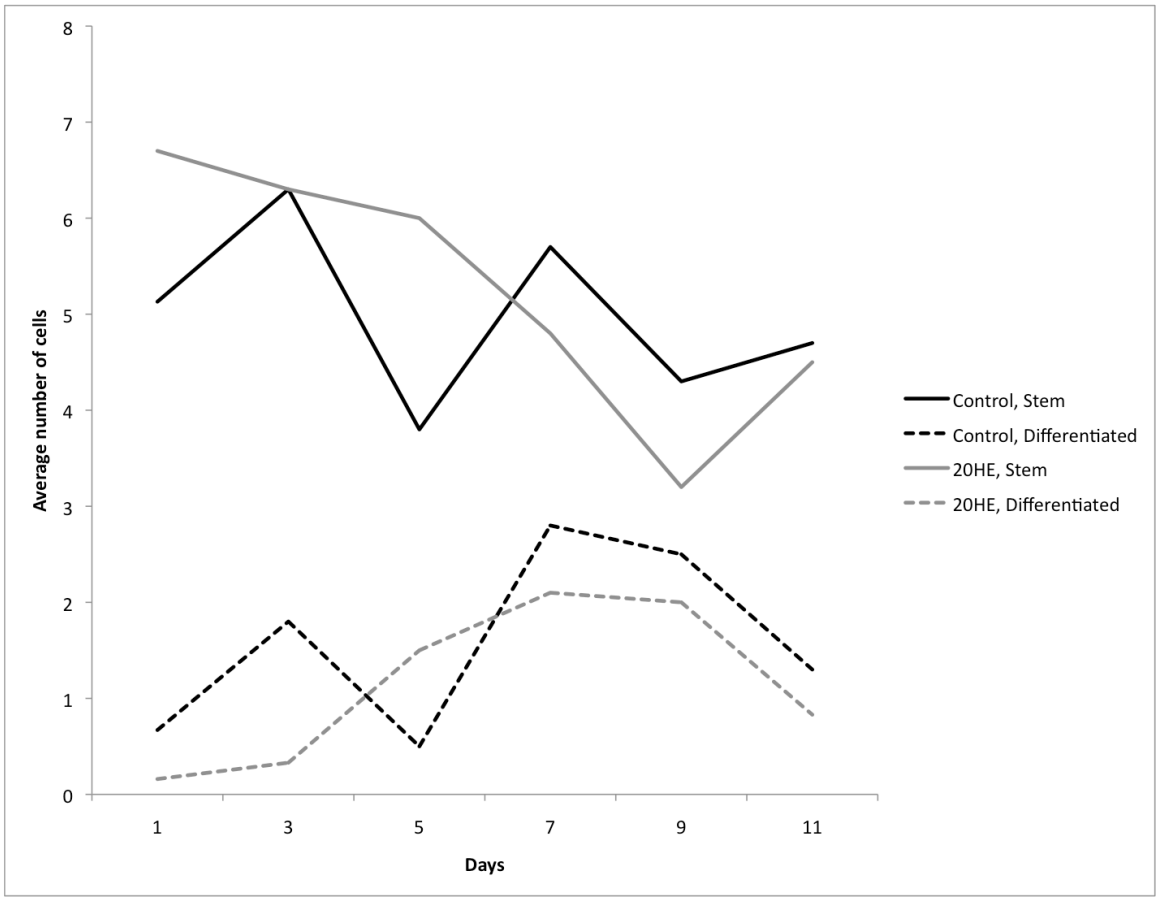

Figure 10. Average number of stem and differentiated cells observed over the lifespan of cultures from newly emerged honey bee donors with and without the addition of 2 µg/ml 20-hydroxyecdysone. Cultures were grown in WH2 media with 1 µg/ml penicillin/streptomycin and 0.25 µg/ml Amphotericin B, n = 12. The average number of cells in culture was not significantly different between cell morphologies. Addition of 20HE did not impact cell numbers or morphological type.

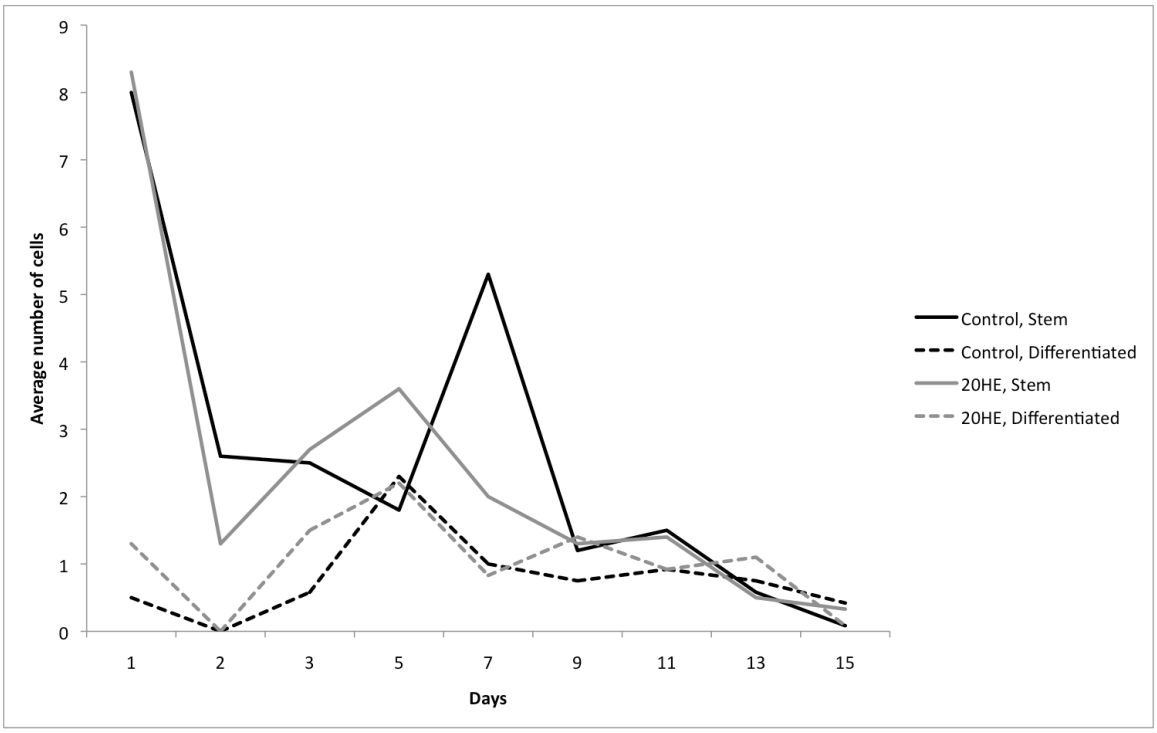

Figure 11. Average number of stem and differentiated cells observed over the lifespan of cultures from nurse honey bee donors with and without the addition of 2 µg/ml 20-hydroxyecdysone. Cultures were grown in WH2 media with 1 µg/ml penicillin/streptomycin and 0.25 µg/ml Amphotericin B, n = 14. The average number of cells in culture was not significantly different between cell morphologies. Addition of 20HE did not impact cell numbers or morphological type.

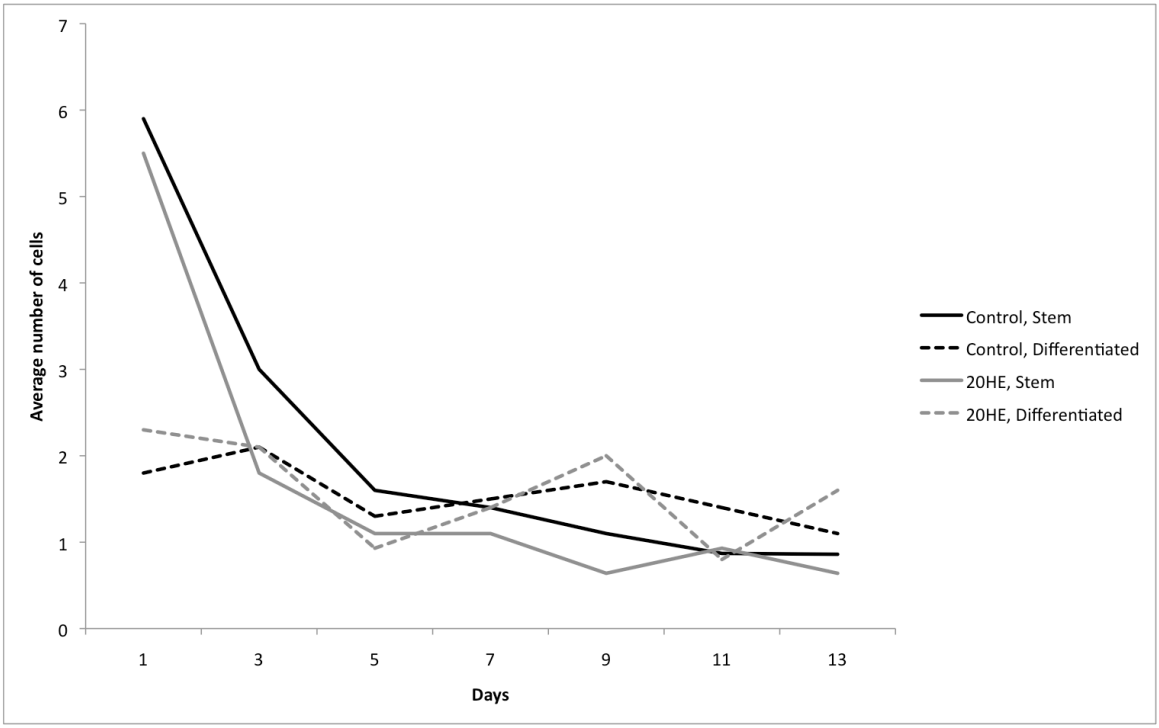

Figure 12. Average number of stem and differentiated cells observed over the lifespan of cultures from forager honey bee donors with and without the addition of 2 µg/ml 20-hydroxyecdysone. Cultures were grown in WH2 media with 1 µg/ml penicillin/streptomycin and 0.25 µg/ml Amphotericin B, n = 9. The average number of cells in culture was not significantly different between cell morphologies. Addition of 20HE did not impact cell numbers or morphological type.

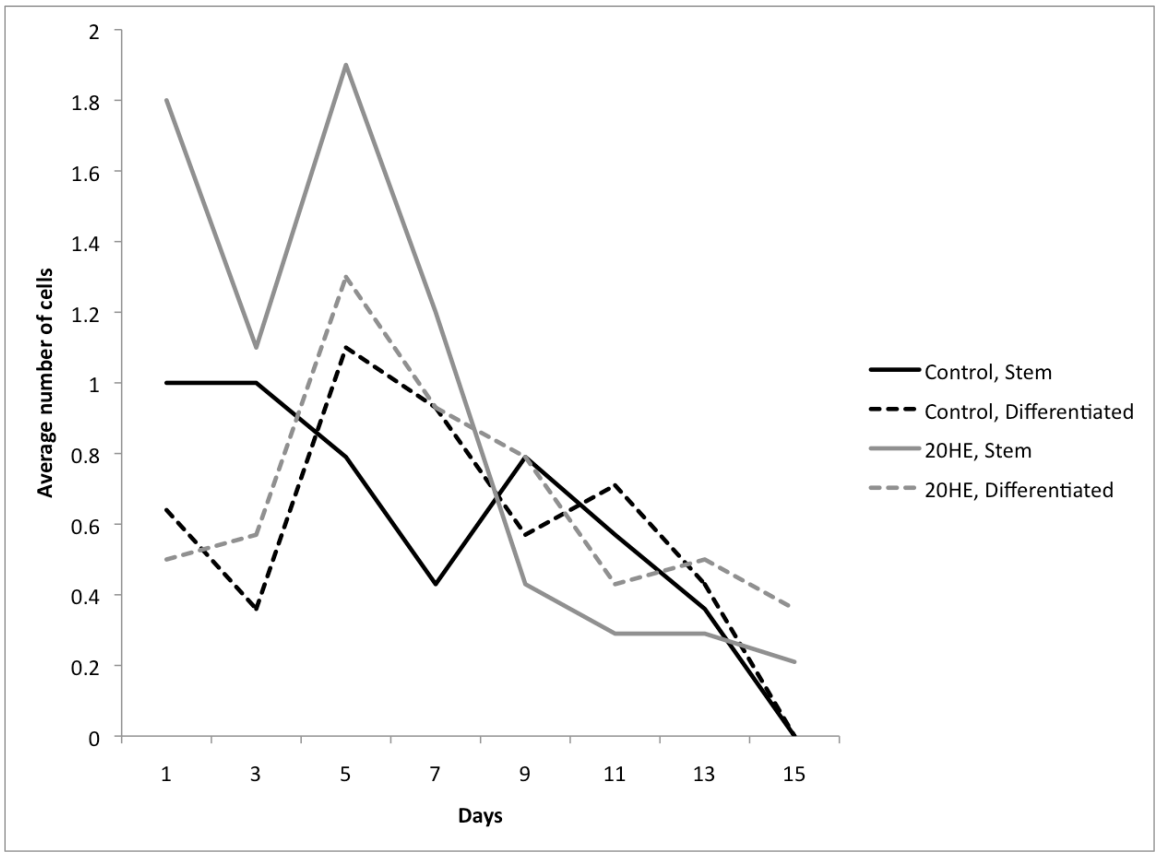

Figure 13. Distinguishing healthy and unhealthy cells with microscopy. Round, healthy cells were easily differentiated from unhealthy cells (red arrow), which were irregular and amorphous.

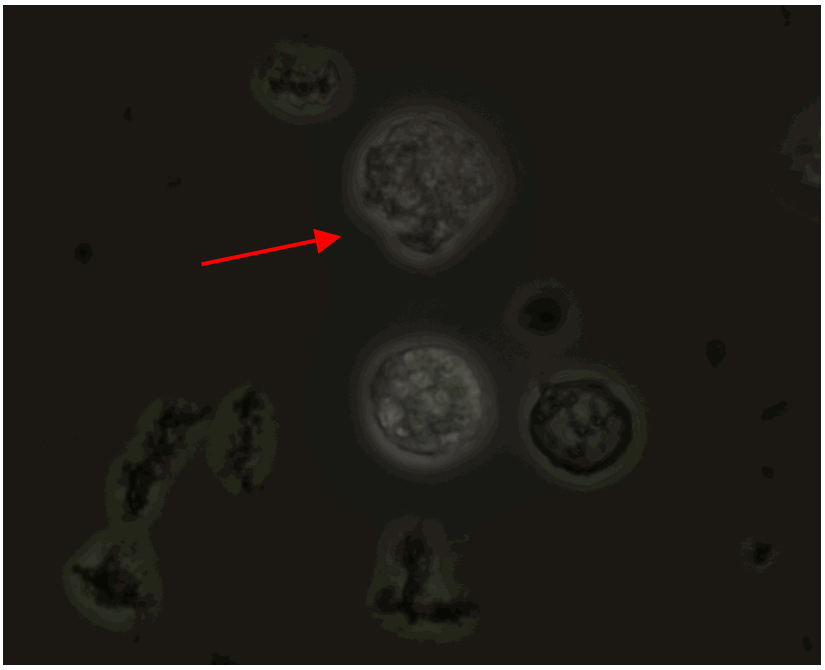

Figure 14. Variation in cell size. Cells in culture varied in size, as seen in these round cells (red arrow).

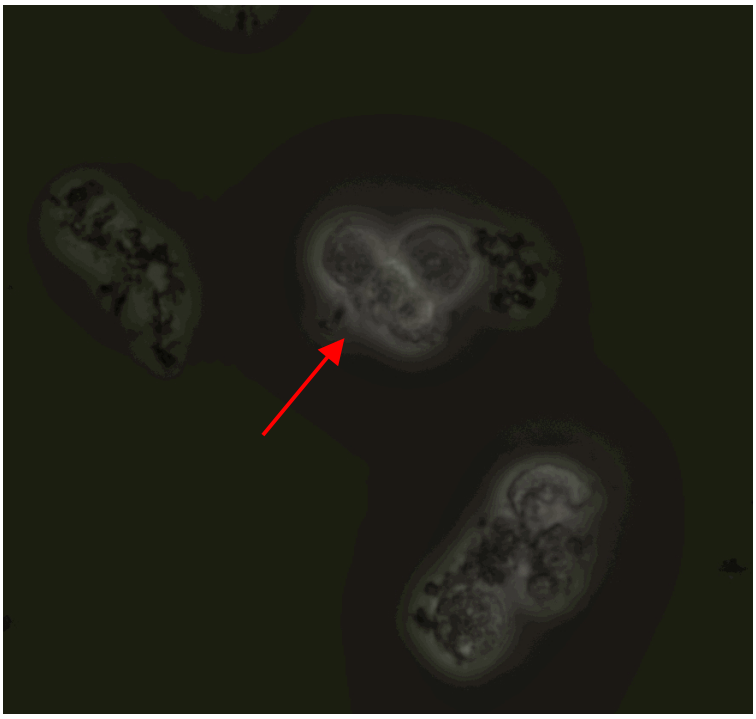

Figure 15. Potential columnar cells in culture. Cells that were rectangular in shape with large central nuclei had an appearance consistent with that of columnar cells. These cells were consistent in size and shape and most often found attached to each other.

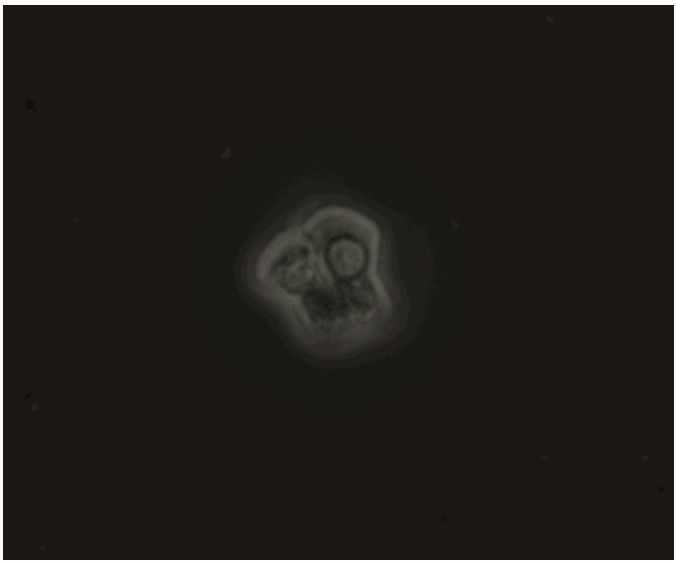

Figure 16. Potential goblet cell in culture. Occasional cells were oblong with thin projections, an appearance consistent with goblet cells.

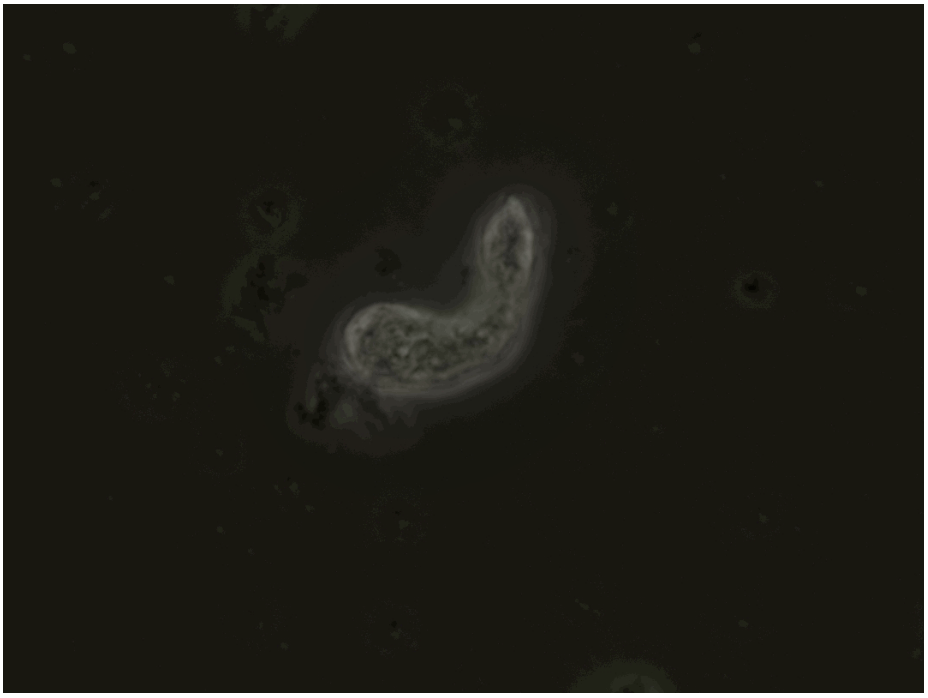

Figure 17. Potential stem cell undergoing cell division. An potential intestinal stem cell (red arrow) appearing to be undergoing cell division in a one-day-old culture.